

**Polymorphisms and Cardiovascular Disease**

# The T<sup>-786</sup>C Endothelial Nitric Oxide Synthase Genotype Is a Novel Risk Factor for Coronary Artery Disease in Caucasian Patients of the GENICA Study

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<b>OBJECTIVES</b>	We investigated the association of polymorphisms in the promoter region and exon 7 endothelial nitric oxide synthase (eNOS) gene with coronary artery disease (CAD).
<b>BACKGROUND</b>	Endothelial dysfunction foretells cardiovascular events and can be genetically determined.
<b>METHODS</b>	We genotyped for the promoter (T <sup>-786</sup> C) and exon 7 (Glu298Asp, G <sup>894</sup> T) polymorphisms in 1,225 subjects; 1,106 were consecutive patients undergoing coronary angiography and 119 control subjects without any cardiovascular risk factors. Genotyping was performed with melting curve analysis of polymerase chain reaction products from allele-specific acceptor and donor probes that were 5'- and 3'-end labeled with LCRed640 and fluorescein, respectively; CAD was assessed by quantitative coronary angiography. We performed multiple logistic regression analysis for the effect of the T <sup>-786</sup> C, the missense Glu298Asp variant, and other coronary risk factors on two- and three-vessel CAD.
<b>RESULTS</b>	The overall genotype distribution of T <sup>-786</sup> C (CC = 17.7%, CT = 40.4%, and TT = 41.9%) and Glu298Asp (GG = 43.3%, GT = 37.0%, and TT = 19.7%) was consistent with the Hardy-Weinberg equilibrium. The regression analysis showed that the T <sup>-786</sup> C, but not the missense Glu298Asp variant, significantly predicted CAD, independent of other risk factors. Compared with TT homozygous, subjects carrying the C allele had a significant (p = 0.002) increase in the odds ratio of harboring two- or three-vessel CAD of 1.672 (95% confidence interval, 1.062 to 2.527). A subgroup analysis confirmed this effect of the T <sup>-786</sup> C polymorphism in men (p = 0.007), cigarette smokers (p = 0.001), subjects older than 60 years of age (p = 0.007), with hypercholesterolemia (p = 0.011), low high-density lipoprotein cholesterol (p = 0.006), and overweight or with obesity (p = 0.041).
<b>CONCLUSIONS</b>	The C allele at the T <sup>-786</sup> C endothelial nitric oxide synthase polymorphism is associated with a higher risk of multivessel CAD in Caucasians. (J Am Coll Cardiol 2003;41:930-7) © 2003 by the American College of Cardiology Foundation

Coronary artery disease (CAD), the leading cause of mortality in Western countries (1), is a highly prevalent heterogeneous disease that is genetically determined, albeit in a complex fashion. Genetic mechanisms are deemed to increase susceptibility to CAD and may account for a sub-

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stantial proportion of CAD variance, but, despite intensive investigation, their identification has not been accomplished thus far (2). This task proved to be unduly difficult for several reasons, including the fact that the clinical identification of the phenotype CAD can be inaccurate without

undertaking coronary angiography. The latter is invasive, costly, inconvenient to the patients, and carries a small, but not negligible, risk of complications (3); therefore, it was not widely used in large studies. Instead, cardiovascular (CV) events, including myocardial infarction (MI), and sudden death, angina, need of coronary artery bypass surgery and/or percutaneous transluminal coronary angioplasty, and evidence for myocardial ischemia at noninvasive tests, were used as surrogates of CAD based on the assumption that they could mirror the presence of CAD. These crude phenotype definitions might well account for the negative or conflicting results that were accomplished.

An impaired endothelium-dependent vasodilation (4) has been consistently reported in conditions that are associated with accelerated atherosclerosis, such as arterial hypertension, cigarette smoking, diabetes mellitus, hypercholesterolemia, hyperhomocyst(e)inemia, and aging (5,6). Furthermore, a blunted endothelium-dependent vasodilation was found to predict CV events independently of the common risk factors (7) and, therefore, might be a precursor of CAD.

Nitric oxide (NO) is a major mediator of endothelium-

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#### Abbreviations and Acronyms

CAD	= coronary artery disease
CV	= cardiovascular
eNOS	= endothelial nitric oxide synthase
GENICA	= Genetic and ENvironmental Factors in Coronary Atherosclerosis study
HDL	= high-density lipoprotein
LDL	= low-density lipoprotein
MI	= myocardial infarction
NO	= nitric oxide

dependent vasodilation made in endothelial cells from L-arginine through the action of the homodimeric enzyme endothelial nitric oxide synthase (eNOS). In addition to its key role in the regulation of vascular tone and blood pressure (8,9), NO is thought to be involved in atherogenesis (8,10,11), development of heart failure, and congenital septal defects and vascular remodeling, as shown by data in mice lacking the eNOS gene (10,12). In fact, NO blunts the activity of the nuclear factor-kappaB family of transcription factors (13,14) and, thereby, can prevent the endothelial expression of adhesion molecules and inflammatory cytokines, which are instrumental for the triggering of atherogenesis (15). Accordingly, a genetically determined impaired availability of NO might be a major risk factor for development of CAD.

The eNOS gene (16) is expressionally and functionally regulated through multiple regulatory steps (14,17) and entails several polymorphisms (18), some of which bear functional consequences. Therefore, this gene is a likely candidate for impaired endothelium-dependent vasodilation and CAD. Some eNOS gene polymorphisms have indeed been linked to CV phenotypes and events (19–28), and two of them to coronary spasm (23,29). Of the latter, the T<sup>-786</sup>C is located in the promoter region, which is the most important for the regulation of the transcription rate of the eNOS gene (29). The mutant C allele implies a blunted transcription rate and was found to be far more common in Japanese patients with vasospastic angina than in controls (29); furthermore, we recently reported that this allele was associated with a blunted forearm vasodilation in response to acetylcholine in hypertensive patients (30).

Another eNOS polymorphism, the GAG to GAT substitution in exon 7, results in the replacement of glutamate by aspartate (Glu298Asp, G<sup>894</sup>T), which might impair the function of eNOS by causing a tight turn of the alpha-helix (23), as suggested by some studies (31–34).

As both polymorphisms were found to be associated with an altered coronary vasomotor reactivity and with an impaired endothelium-dependent vasodilation (29,30), we hypothesized that they might play a role for the triggering of atherogenesis and CAD occurrence. However, only limited and conflicting results were available so far to support this hypothesis. No association of the T<sup>-786</sup>C polymorphism with CV events (35) and of the Glu298Asp polymorphism

with MI was seen in Caucasians (36). However, both these studies relied on CV events and not on coronary angiography for the assessment of CAD.

Thus, within the Genetic and ENvironmental factors In Coronary Atherosclerosis (GENICA) study, a large prospective investigation of the genetic determinants of CAD, which was performed in consecutive Caucasian patients undergoing coronary angiography, we sought to determine if the T<sup>-786</sup>C and the Glu298Asp polymorphisms predicted multivessel CAD.

## METHODS

The criteria for enrollment of the patients and controls in the GENICA study were previously reported (37). In brief, consecutive Caucasian patients of both genders consecutively referred to the Division of Cardiology of the Citta-della General Hospital for coronary angiography for investigation of chest pain and/or suspected CAD were enrolled between 1999 and 2001. The Medical Ethics Committee of our university approved the study protocol, and a written consent after explanation of the aims and details of the study was obtained from each participant. The refusal to participate in this study was the only exclusion criterion. Two groups served as controls: group 1 entailed patients in whom significant (e.g., stenosis  $\geq 50\%$ ) CAD was eventually ruled out by coronary angiography; group 2 comprised 119 consecutive healthy normotensive blood donors enrolled at the local blood bank during the same period. In these latter subjects, it was unethical to perform coronary angiography to rule out the presence of asymptomatic CAD. Therefore, the following inclusion criteria were used: negative family history of CAD, MI, and stroke; nonsmoking status; absence of hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, all defined as specified in the following text. Based on available data from epidemiologic and family studies, a cohort fulfilling these criteria is expected to have a very low prevalence of asymptomatic CAD.

**Demographic and clinical measurements.** A standard questionnaire was used to carefully ascertain medical history in all participants (transient ischemic attack, stroke, angina, MI, coronary artery bypass, percutaneous transluminal coronary angioplasty, renal failure, peripheral artery disease, and history of vascular surgical interventions), smoking habits, presence/absence of hypertension, diabetes, hypercholesterolemia, hypertriglyceridemia, and current medications. Body mass index was calculated as weight/height<sup>2</sup> (kg/m<sup>2</sup>). Patients were classified into three groups: current smokers, nonsmokers, and ex-smokers (who had stopped smoking for at least one year). Diabetes mellitus (type I or II) was defined as a previous diagnosis of the disease, history of antidiabetic medications, or plasma fasting levels of glucose  $\geq 126$  mg/dl (7.0 mmol/l) on at least two occasions. Impaired glucose tolerance was defined as plasma fasting levels of glucose ranging between 110 to 126 mg/dl (6.1 to 6.9 mmol/l) (38). Hypercholesterolemia was defined as a

low-density lipoprotein (LDL) cholesterol  $\geq 100$  mg/dl according to the National Cholesterol Education Program guidelines for patients with CAD (39); hypertriglyceridemia was defined as plasma fasting levels  $\geq 134$  mg/dl, that is, higher than the 95th percentile value of our group 1 control subjects. Blood pressure was measured by mercury sphygmomanometer using Korotkoff phase V for diastolic, according to the World Health Organization guidelines. Hypertension was defined as systolic pressure  $\geq 140$  mm Hg, and/or diastolic pressure  $\geq 90$  mm Hg (40), or use of any antihypertensive agents.

**Coronary angiography.** Angiography was carried out and evaluated by experienced cardiologists who were blinded to patients' genotype. The severity of CAD was determined by the number of significantly stenosed coronary arteries (41). Patients were classified as follows: code 1 = normal vessels; code 2 =  $<50\%$  stenosis; code 3, 4, and 5 = stenosis  $\geq 50\%$  in one, two, or three major coronary arteries, respectively.

**Laboratory measurements.** Each patient was studied between 8:30 and 12:00 A.M. Before coronary angiography, blood samples were taken from the femoral artery and were immediately put on ice and centrifuged at  $3,000 \times g$  (at  $4^\circ\text{C}$  for 10 min). Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glycemia, sodium, potassium, blood urea nitrogen, and creatinine levels were measured with conventional methods.

**Extraction of deoxyribonucleic acid and eNOS genotyping.** The blood was stored at  $-20^\circ\text{C}$  until deoxyribonucleic acid was extracted using a commercially available kit (DNA Blood Extraction Fast KIT, Analitica Srl., Padova, Italy). Details of the methodology used for genotyping at each polymorphism, including oligonucleotides serving as amplification primers and fluorescence resonance energy transfer probes, were recently reported (30).

**Statistical analysis.** One-way analysis of variance followed by Bonferroni's post-hoc test was used to compare quantitative variables between CAD patients and control subjects. Chi-squared analysis was used to compare the frequencies of the categorical coronary risk factors and of eNOS gene variants between the CAD and the control groups. Consistency of the genotype frequencies with the Hardy-Weinberg equilibrium was also tested by chi-squared analysis. To identify the independent risk factors for CAD, we performed multiple logistic regression analysis for the effect of the  $T^{-786}C$ , the missense Glu298Asp variant, and the other coronary risk factors. To this end, independent variables were coded as dummy variables as follows: age, 0 for  $<60$  years, 1 for  $\geq 60$  years; gender, 0 for female, 1 for male; body mass index, 0 for  $<26$  kg/m<sup>2</sup>, 1 for  $\geq 26$  kg/m<sup>2</sup>; hypertension, diabetes mellitus, and LDL cholesterol,  $\geq 100$  mg/dl, 0 for absence, 1 for presence; cigarette smoking, 0 for nonsmoker, 1 for current or ex-smoker; the  $T^{-786}C$ , 0 for CC, 1 for CT, and 2 for TT; missense Glu298Asp variant, 0 for GG, 1 for TG, and 2 for TT (42). Because the aim of this study was to identify predictors of multivessel CAD, the dependent variable was coded as follows: 0 for normal

coronary artery and for stenosis  $<50\%$ ; 1 for stenosis  $\geq 50\%$  in two or three major epicardial vessels. The forward stepwise selection (Wald criterion) was used. Odds ratios (approximating to relative risks) were calculated as a measure of the association between the eNOS genotype and the CAD phenotype, with the effect of the mutant allele C for the  $T^{-786}C$  polymorphism assumed to be dominant (TT vs. CT and CC) or additive (TT and CT combined vs. CC). Similarly, for the Glu298Asp polymorphism, the effect of the mutant allele was investigated according to a dominant (TT and TG combined vs. GG) or additive (TT vs. GT and GG) model of inheritance. For each odds ratio, we calculated two-tailed p values and 95% confidence intervals. Statistical significance was defined as  $p < 0.05$ . All analyses were performed using SPSS 10.0 for Windows (SPSS Italy Inc., Bologna, Italy).

## RESULTS

**Demographic characteristics and eNOS genotype distribution.** Of the 1,271 patients originally recruited in the GENICA study who had complete coronary angiography data, 17% ( $n = 217$ ) were found to have normal coronary arteries, and 14% ( $n = 178$ ) stenosis  $<50\%$ , 23% ( $n = 290$ ), 24% ( $n = 305$ ), and 22% ( $n = 281$ ) had significant ( $\geq 50\%$ ) stenosis in one, two, or three major epicardial vessels, respectively. The eNOS polymorphism genotypes were available in 1,106 (87%) of all patients. A preliminary regression analysis on all subjects revealed a borderline significant ( $p = 0.034$ ) association between the  $T^{-786}C$  eNOS genotype and one-, two-, or three-vessel CAD. Therefore, to maximize contrast at the phenotype level, it was decided to combine two- and three-vessel CAD patients, as CAD group, and normal vessel and  $<50\%$  stenosis as control group (group 1); thus, the CAD group and group 1 comprised 749 and 330 patients, respectively. Of the latter, 21% were healthy individuals, 39% had valvular heart disease, 15% had cardiomyopathy (13.7% dilated, 1.3% hypertrophic), 7% had hypertensive heart disease, and 18% had ischemic cardiomyopathy. The latter diagnosis was based on resting and stress electrocardiogram and on results of stress myocardial scintigraphy that showed inducible ischemia.

The demographic and clinical characteristics of the CAD patients and of group 1 and 2 (119 subjects) controls are shown in Table 1. The CAD patients were older, had higher plasma glucose and triglycerides, and lower HDL cholesterol than control subjects. There were more males in CAD patients than in group 1 ( $p < 0.001$ ), but not group 2 control subjects. Compared with both control groups, more CAD patients had diabetes mellitus, hypercholesterolemia (both  $p < 0.001$ ), hypertriglyceridemia ( $p < 0.001$ ), and were smokers or ex-smokers ( $p < 0.0001$ ). Compared with controls, the CAD patients more commonly reported a history of myocardial infarction ( $p < 0.0001$ ), coronary artery bypass surgery ( $p < 0.0001$ ), percutaneous translu-

**Table 1.** Clinical Characteristics of the CAD Patients and the Control Subjects

Group Variables	CAD (n = 749)	p vs. Group 1	Controls			
			Group 1 (n = 330)	p vs. Group 2	Group 2 (n = 119)	p vs. CAD
Age, yrs	64.3 ± 9.4	0.001	62.0 ± 10.8	< 0.001	32.8 ± 8.0	< 0.001
Gender M/F (%)	611 (82%)/138 (18%)	< 0.001	191 (58%)/139 (42%)	< 0.001	103 (87%)/16 (13%)	NS
BMI (kg/m <sup>2</sup> )	26.9 ± 3.8	NS	26.8 ± 4.4	< 0.001	23.9 ± 3.6	< 0.001
Systolic BP (mm Hg)	135 ± 18	NS	134 ± 17	< 0.001	119 ± 8	< 0.001
Diastolic BP (mm Hg)	78 ± 10	NS	79 ± 10	< 0.001	70 ± 7	< 0.001
Mean BP (mm Hg)	97 ± 11	NS	97 ± 11	< 0.001	86 ± 7	< 0.001
Heart rate (beats/min)	66 ± 10	NS	69 ± 10	NS	NA	NS
Total cholesterol (mg/dl)	206 ± 44	NS	202 ± 41	< 0.001	175 ± 22	< 0.001
HDL cholesterol (mg/dl)	45 ± 11	< 0.001	48 ± 13	–	NA	–
LDL cholesterol (mg/dl)	131 ± 36	NS	129 ± 34	–	NA	–
Triglycerides (mg/dl)	147 ± 98	< 0.001	125 ± 71	< 0.001	73 ± 35	< 0.001
Glycemia (mg/dl)	115 ± 40	< 0.001	108 ± 25	< 0.001	88 ± 8	< 0.001
Left ventricular ejection fraction (%)	60 ± 14	NS	60 ± 16	–	NA	–
Diabetes mellitus (%)	19.0	0.004	11.5	< 0.001	0	< 0.001
Nonsmoker/smokers/ex-smokers (%)	35.0/14.2/50.8	< 0.001	59.1/14.3/26.5	< 0.001	100/0/0	< 0.001
Hypertensives (%)	61.4	NS	56.6	< 0.001	0	< 0.001
Hypercholesterolemia (%)	59.4	< 0.001	46.1	< 0.001	0	< 0.001
Hypertriglyceridemia (%)	22.7	< 0.001	13.3	< 0.001	0	< 0.001
Cholesterol-lowering drugs (%)	38.6	< 0.001	18.8	< 0.001	0	< 0.001
History of						
Transient ischemic attack (%)	3.5	NS	4.6	0.009	0	0.026
Ischemic stroke (%)	1.7	NS	1.2	NS	0	NS
Acute myocardial infarction (%)	45.6	< 0.001	11.7	< 0.001	0	< 0.001
Coronary artery bypass surgery (%)	12.1	< 0.001	0	NS	0	< 0.001
Percutaneous coronary angioplasty (%)	9.3	< 0.001	0.3	NS	0	< 0.001
Peripheral arterial disease (%)	17.5	0.008	11.7	< 0.001	0	< 0.001
Vascular surgery (%)	5.4	0.036	2.2	0.051	0	0.002
Chronic renal failure (%)	6.7	0.042	3.7	0.027	0	< 0.001

Results are expressed as mean ± SD.

BMI = body mass index; BP = blood pressure; CAD = coronary artery disease; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NA = not available.

minimal coronary angioplasty ( $p < 0.0001$ ), peripheral artery disease ( $p < 0.001$ ), vascular surgery ( $p < 0.001$ ), but not of transient ischemic attack or stroke.

**Screening for the T<sup>-786</sup>C polymorphism and the missense Glu298Asp variant of the eNOS gene.** The overall genotype distribution of the T<sup>-786</sup>C polymorphism was CC = 17.7%, CT = 40.4%, and TT = 41.9%; the C and T allele frequency was 0.38 and 0.62, respectively. For the Glu298Asp polymorphism, the genotype distribution was GG = 43.3%, GT = 37.0%, and TT = 19.7%; the G and T allele frequency was 0.62 and 0.38, respectively. The distribution of both polymorphisms agreed with the Hardy-Weinberg equilibrium; it did not differ between CAD and control patients for the T<sup>-786</sup>C polymorphism. The frequency of the Glu298Asp genotypes did not differ between CAD patients and group 1 controls, whereas, in group 2, there was a slight excess of GG homozygous ( $p = 0.015$ ). For both polymorphisms, all demographic and clinical variables did not differ between genotypes.

We investigated whether there was a linkage between the T<sup>-786</sup>C and the Glu298Asp polymorphism, by Kendall's tau b and Spearman's correlation analysis, and found a highly significant ( $p < 0.001$ ) relationship between the two polymorphisms (Kendall's tau b =  $0.303 \pm 0.025$ ; Spearman's correlation =  $0.330 \pm 0.027$ ).

**Association between T<sup>-786</sup>C, Glu298Asp, eNOS variants, and CAD.** We found with the logistic regression analysis that the T<sup>-786</sup>C polymorphism significantly predicted CAD. In Table 2 we ranked the variables that entered into the model according to the value of the Wald coefficient, which estimates the usefulness of the parameter to the model. Besides confirming that male gender, age, hypertriglyceridemia, and cigarette smoking were significant predictors of CAD, we found that the C allele also significantly predicted CAD, being as useful as cigarette smoking to this end. At variance, other "classical" CV risk factors, such as arterial hypertension, diabetes mellitus, obesity, and elevated LDL cholesterol, did not remain in the model and, therefore, were less useful than the C allele as predictors of CAD. Subjects carrying the C allele, either homozygous or heterozygous, had a 69.2% increased risk of having significant multivessel CAD. We observed similar results when the C allele was examined according to an additive model, that is, CC homozygous versus CT and TT (Table 2). In contrast, no effect of the Glu298Asp missense variant on CAD was detected. The association of the C allele with multivessel CAD was even stronger ( $p < 0.001$ ) when the 18% of group 1 control patients with ischemic cardiomyopathy were excluded from the analysis.

Subgroup analysis (Fig. 1) confirmed the association of

**Table 2.** Results of Logistic Multivariate Analysis for T<sup>-786</sup>C

Variables in the Model	B	SE	Wald	p Value	OR (95% CI)
Gender	-1.201	0.210	32.78	< 0.0001	0.301 (0.199-0.454)
Age	-0.654	0.179	13.396	< 0.0001	0.520 (0.366-0.738)
Hypertriglyceridemia	-0.559	0.176	10.122	0.001	0.572 (0.405-0.807)
T <sup>-786</sup> variant (C dominant)	0.546	0.172	10.112	0.001	1.727 (1.233-2.419)
Cigarette smoking	-0.546	0.186	8.635	0.003	0.579 (0.402-0.834)
Diabetes mellitus	-0.551	0.206	7.154	0.007	0.576 (0.385-0.863)
T <sup>-786</sup> variant (C additive)	0.521	0.211	6.094	0.014	1.684 (1.113-2.546)

The following variables did not enter into the model: arterial hypertension, low-density lipoprotein cholesterol > 100 mg/dl, Glu298Asp polymorphism (both dominant and codominant).

B = estimated coefficient; CI = confidence interval; OR = odds ratio; SE = standard error.

the C allele with CAD also when men ( $p = 0.007$ ), subjects older than 60 years of age ( $p = 0.007$ ), subjects with hypercholesterolemia ( $p = 0.011$ ), cigarette smokers ( $p = 0.001$ ), subjects with low HDL cholesterol ( $p = 0.006$ ), and overweight or with obesity ( $p = 0.041$ ) were considered. When subjects with at least four such unfavorable features were considered, the odds ratio associated with the C allele further increased the relative risk (odds ratio = 3.61; 95% confidence interval, 1.63 to 8;  $p = 0.002$ ) of having significant multivessel CAD.

## DISCUSSION

This study showed a significant association between severe CAD and the T<sup>-786</sup>C polymorphism, located in the eNOS gene promoter, independently of the most common CV risk factors. At variance, we could find no evidence for such association with the Glu298Asp polymorphism in exon 7. Thus, our results identified a genetic determinant of CAD in Caucasian individuals.

Reporter gene studies showed that the T<sup>-786</sup>C substitution in the promoter region of eNOS reduced by 50% the rate of transcription of eNOS, both under baseline conditions and in response to hypoxia (29), and was associated with decreased serum levels of nitrite/nitrate (43). These effects might depend upon the fact that a mutant allele can bind the replication protein A1, which acts as a gene repressor protein (43). The mutant C allele was found to be quite rare in healthy Japanese subjects (3%) and more common (15%) in patients with coronary spasm, thus indicating a highly significant ( $p < 0.0001$ ) association with an altered coronary vasomotor responses in this ethnic group (44). We found the C allele in 38% of our cohort, which is consistent with the 37.8% recently reported in British middle-age men from primary care practices (35). Thus, this allele seems to be much more common in Caucasian than in Japanese individuals, albeit the underlying reasons remain hypothetical.

To our knowledge there was no data supporting the relevance of the T<sup>-786</sup>C polymorphism in non-Japanese populations, because no association with CAD was found in one study (35). Our results filled this gap by showing that the C allele was associated with CAD in Caucasian subjects. The logistic regression (Table 2) showed that subjects who carried the C allele exhibited a 73% increase of the risk of

harboring significant multivessel coronary artery narrowing. This significant association does not imply, per se, a causal relationship; neither does it clarify the basic mechanisms. However, it is worth pointing out that, in a large series of mild-to-moderate uncomplicated essential hypertensive patients who underwent an infusion of the endothelium-dependent vasodilator acetylcholine into the brachial artery, the TT homozygous subjects exhibited a significantly enhanced vasodilation, as compared with CT and CC individuals (30). Thus, it would appear that, in a condition that implies enhanced oxidative stress, such as arterial hypertension, the genetic predisposition to generate less NO can become apparent and, in the long run, might be detrimental and result in susceptibility to atherogenesis.

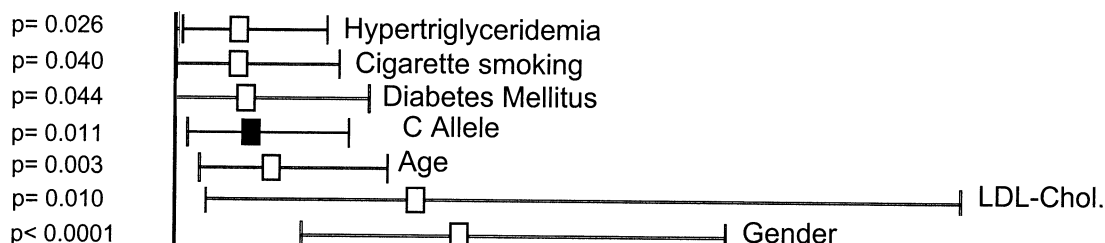
This contention is further supported by the present subgroup analysis. We found that the concomitance of at least one major CV risk factor further enhanced the additional risk associated with the C allele and that the concurrence of multiple risk factors markedly enhanced the nefarious effects of the C allele (Fig. 1). Thus, under most conditions that imply oxidative stress, such as aging, smoking, hypercholesterolemia, male gender (lack of estrogens), and overweight or obesity, the genetic predisposition to generate less NO associated with the C allele could contribute to decreasing the bioavailability of NO and, thus, to the triggering of coronary atherogenesis.

Recent studies showed an association of the T (mutant) allele at the Glu298Asp polymorphism with human phenotypes that can be relevant for CV disease, including vascular adaptation to pregnancy (34), endothelial response to smoking and n-3 fatty acids (32), high-altitude pulmonary edema (33), and a lower mean age at end-stage renal disease in patients with autosomal dominant polycystic kidney disease (31). We found that frequency of the T (mutant) allele was 38%, that is, much higher than in Japanese (6.7% to 10.7% in a study [42], 9.8% to 25.6% in another study [33]), but similar to that observed in Caucasians from continental Europe (31% to 34%) (31,45), albeit lower than in U.K. patients (68.8% and 52.2% in control subjects and CAD patients, respectively) (36). We found no evidence for an association of this polymorphism with severity of CAD in agreement with earlier and more recent studies (26,35,42,45,46) but in contrast to reports of an association of the Glu298 with MI (36,42). This might not be surpris-

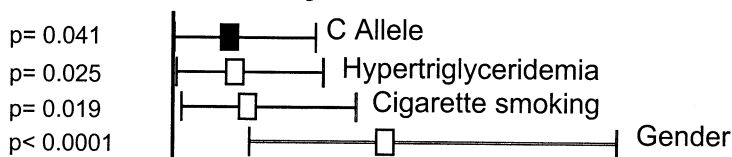
## 4 Major Risk Factors



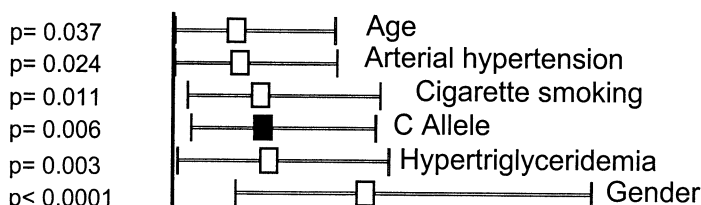
## Hypercholesterolemia



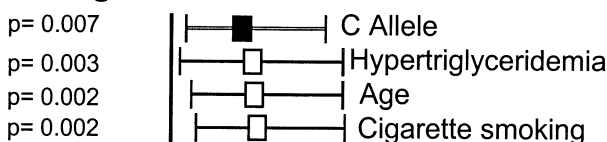
## Overweight-Obesity



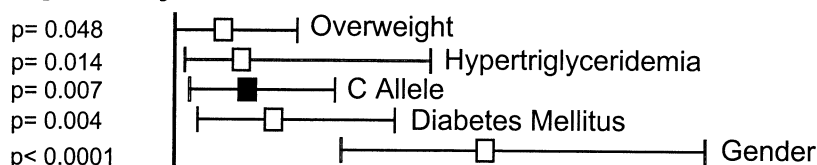
## Low HDL Cholesterol



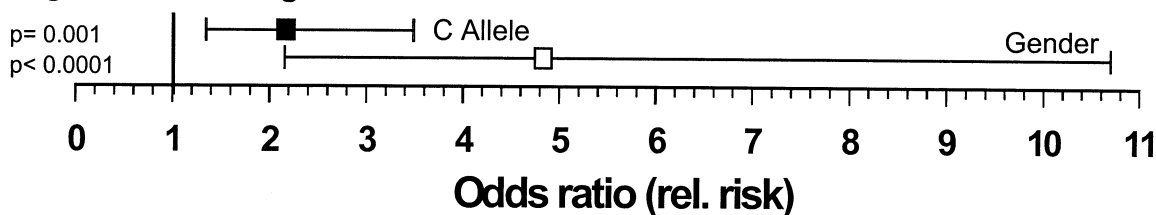
## Male gender



## Age > 60 yrs



## Cigarette smoking



**Figure 1.** Results of the logistic regression analysis in the different risk subgroups of subjects and in subjects with concurrence of at least four major risk factors (**top**). The **squares** (closed for the T<sup>-786</sup>C C allele; open for the other variables) and the **lines** indicate, respectively, the value of the B coefficient (an estimate of the odds ratio) and its 95% confidence interval. For the purpose of visual clarity, the reciprocal value of the B coefficient that was <1 was used. The figure shows the independent variables that remained in the model, that is, were significant predictors of multivessel coronary artery disease (CAD) in each subgroup, ranked from **bottom to top** according to the value of the Wald coefficient. Notice the usefulness of T<sup>-786</sup>C polymorphism for the prediction of multivessel CAD in all subgroups. The coexistence of four major risk factors (subgroup at the **top**) resulted in a clear-cut increase of the odds ratio for C allele of the T<sup>-786</sup>C polymorphism, further confirming the usefulness of this endothelial nitric oxide synthase molecular variant for the prediction of multivessel CAD. HDL = high-density lipoprotein; LDL = low-density lipoprotein.

ing because: 1) the replacement of aspartate for glutamate is deemed to be a conservative one, and 2) recombinant eNOS Asp298 and Glu298 enzymes showed no discernible difference in the Michaelis constant ( $K_m$ ) or the maximum velocity ( $V_{max}$ ) (18). However, at variance with this latter finding, it was found that the enzymatic activity of eNOS was systematically decreased in renal artery specimens of patients harboring the T allele (31). Thus, either the functional impact of this polymorphism can differ between the renal and the coronary artery bed or this polymorphism could only act as a marker for a functional locus elsewhere in the gene, as suggested by the observation of a linkage disequilibrium with other eNOS polymorphisms (35). Of much interest, we found evidence of a significant linkage between T<sup>-786</sup>C and the Glu298Asp polymorphism, at variance with a previous report (47). Thus, available conflicting results on the association of the Glu298Asp polymorphism with CAD or CV events might be explained on the basis of a variable association of this variant with the T<sup>-786</sup>C polymorphism in different populations. However, individuals carrying the T allele (TT and TG) were found to have significantly higher pressor responses to phenylephrine than GG homozygous, thus suggesting that the T allele might increase vascular reactivity (48), possibly because the Asp298, but not the Glu298, variant of eNOS is susceptible to cleavage (49). Whatever the functional relevance of the missense Glu298Asp variant might be, our results in individuals from southern Europe, consistent with earlier studies (26,35,42), do not support the contention that Glu298Asp polymorphism is, per se, strongly associated with CAD in Caucasian patients.

This cross-sectional study comprised exclusively Caucasians, and, therefore, our findings might not apply to other ethnic groups or indicate a prognostic value of the T<sup>-786</sup>C polymorphism, which might be revealed by an ongoing follow-up study. Furthermore, only a minority of our study population was women, while estrogens are known to enhance NO availability and to account for protection of fertile women from CV events. We could confirm the usefulness of the T<sup>-786</sup>C polymorphism as a predictor of CAD in men but not in women; however, we cannot exclude the possibility that this study was underpowered to detect an association of the T<sup>-786</sup>C eNOS polymorphism with CAD in both genders.

In conclusion, we have found that a functional polymorphism of the eNOS gene, the T<sup>-786</sup>C, which is located in the promoter region, was significantly associated with severe CAD, independent of the other common CV risk factors in Caucasian patients.

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